

The role of structural domains in RIP II toxin model membrane binding

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Abstract The interaction of plant toxin ricin and MLI binding subunits to liposomes containing monosialoganglioside (GM1), bearing a terminal galactose residue, has been examined as a possible receptor model. For the first time we demonstrate that ricin B-chain but not ricin provokes liposome aggregation at 10 M% GM1 concentration, whereas in the presence of either ricin A-chain or galactose the aggregation is inhibited. The B-subunit of plant toxin MLI from *Viscum album* has similar lectin specificity and activity but cannot aggregate GM1 liposomes. The ability of the B-chain to aggregate liposomes adds a new crucial step in the toxin transmembrane penetration mechanism. We demonstrate here possible ricin B-chain interactions with membranes proceeding via two sites, namely (a) a galactose-binding domain and (b) a hydrophobic interchain domain. In close contact with two phospholipid bilayers, ricin B-chain may determine the geometry of the fusion site. These events can provoke A-chain translocation which follows membrane fusion.

Key words: Ricin; Ricin B-chain; Mistletoe lectin I; Liposome aggregation

1. Introduction

The plant toxin ricin, a type II ribosome-inactivating protein (RIP II), comprises two polypeptide chains, A and B, linked by a disulfide bond [1]. The A-subunit (RTA) is a highly specific *N*-glycosidase that modifies 28S RNA of the 60S ribosome subunit. Ricin B-chain (RTB) is a galactose-specific lectin, with two carbohydrate-binding sites which have been characterized structurally by X-ray crystallographic studies at 2.5 Å resolution [2]. The B-chain not only helps to locate the A-chain prior to release, by binding to carbohydrates on the cell surface, but also plays an essential role in promoting the transmembrane penetration of the A-chain into the cytosol [3]. During this process both the A- and B-chains interact with phospholipid bilayers to reach the intracellular target [4]. It has been shown that the ricin A-chain translocation is ATP-mediated [5]. The protein components of intracellular organelle membranes are required for RTA translocation [6]. Studies with GM1-containing membrane models demonstrated that native ricin and its A- and B-chains deeply

penetrate the membrane, especially at pH 4.5 [7]. The recombinant chimeric protein obtained by fusing together RTA and a fragment of protein G of the vesicular stomatitis virus interacts with phospholipid vesicles with 15-fold faster kinetics than native RTA at acidic pH [8].

Toxins with intracellular sites of action, like ricin and MLI, are ideal model proteins for studying lipid-protein interactions and protein transmembrane penetration that occur during the internalization of physiologically active molecules. While the toxicity of RIPs II against target cells is well characterized biochemically [1,2], the molecular mechanism of its translocation remains unclear.

In this study ricin and MLI B-chain activity has been examined in lipid bilayers, both with and without toxin receptors. The experiments were performed at both neutral and acid pH in order to model different cell compartments. It has been shown previously that ricin B-chain itself undergoes structural transitions under acid conditions (the mid-point of the transition being at pH 4.5), whereas the ricin A-subunit conformation is not pH dependent [9]. We compared the ability to aggregate GM1 liposomes of RTB and B-chain of plant toxin MLI (MLIB) from *Viscum album*. The structure and mechanism of action of MLI and ricin are similar [1,2]. Cytotoxic activity of the holotoxins was compared according to the ability of the B-chains to interact with liposomes.

2. Materials and methods

Ricin was extracted from *Ricinus communis* seeds together with ricin agglutinin and subsequently isolated and purified [10]. Isolated A- and B-subunits were also prepared as previously described [10]. The A-subunit was additionally passed through a Sepharose 4B column with fixed asialofetuin in order to completely eliminate traces of the B-subunit. MLI was extracted from *Viscum album* leaves and A- and B-subunits were purified as previously described [11]. The purity of these preparations was estimated using gradient SDS-PAGE (7–22%) and tested for cytotoxicity on the T-lymphoblast Jurkat cell line by measuring the inhibition of [³H]thymidine incorporation [10]. RTB-RTA reassociation was prepared as described in [12]. GM1 ganglioside was kindly provided by Dr. N.V. Prokashova of Moscow Cardiology Research Center, Russia.

Small unilamellar liposomes were produced by drying dimyristoylphosphatidylcholine (DMPC) without and with GM1 ganglioside solutions in varying molar ratio, on a rotor evaporator. Subsequently the mixture was treated for 5 min with ultrasound in a buffer containing 5 mM TES, 140 mM NaCl, 1 mM EDTA, at pH 7.5 in a nitrogen atmosphere (50 W, 22–28 kHz) at +4°C and incubated for 2 h at +28°C.

Phase transition and liposome aggregation were monitored from optical density measurements at a wavelength of 350 nm using a UV-VIS spectrophotometer (Perkin & Elmer, Germany) as previously described [10]. The concentration of lipids in liposome solution was 5 mM in phase transition experiments and 0.25 mM in aggregation

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Abbreviations: RTB, ricin B-chain; RTA, ricin A-chain; MLI, mistletoe lectin I; MLIB, mistletoe lectin I B-chain; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DMPC, dimyristoylphosphatidylcholine; IC₅₀, toxin concentration causing 50% inhibition of [³H]thymidine incorporation into cells

experiments. All liposome aggregation experiments were carried out at +28°C.

3. Results and discussion

Fig. 1 shows the absorbency measurement of a DMPC-liposome suspension, in the presence of ricin B-chain, with increasing temperature and different GM1 concentrations. The mid-point of the phase transition corresponds to about 24°C. At GM1 concentrations of 2, 4 and 8 M% a smoothing of the phase transition and an increase of the corresponding temperature range over which it took place were observed. This strongly suggests a cooperative decrease in the ordering of the lipid bilayer [13]. Addition of either ricin or its A-subunit does not cause significant changes in the absorbency of the suspension at the phase transition temperature at pH 7.5 and 4.5 (data not shown). At a ganglioside/phospholipid molar ratio of 10% after the addition of RTB a steep increase of the suspension absorbency was observed, associated with a transition to the liquid crystal phase and indicative of liposome aggregation. Aggregation did not take place when administering 50 mM galactose to the incubation mixture.

The rate of DMPC/GM1-liposome aggregation depends on the concentration of ricin B-chain in the incubation medium (Fig. 2). MLIB does not affect liposome aggregation. Addition of ricin to the liposome suspension up to a concentration of 0.2 mg/ml did not cause liposome aggregation and completely inhibited liposome aggregation induced by RTB (data not shown). Fig. 3 shows the effect of ricin A-chain on RTB-induced DMPC/GM1-liposome aggregation. Ricin B-chain did not provoke liposome aggregation in the presence of RTA because of the dimerization of RTB and RTA (checked by size exclusion HPLC, data not shown). In control experiments the liposome aggregation with B-chain was not affected by the presence of IgG, chymotrypsin or BSA. Data of cytotoxic activity of the holotoxins are shown in Fig. 4. Comparing the IC_{50} value the native ricin is 25 times more toxic than

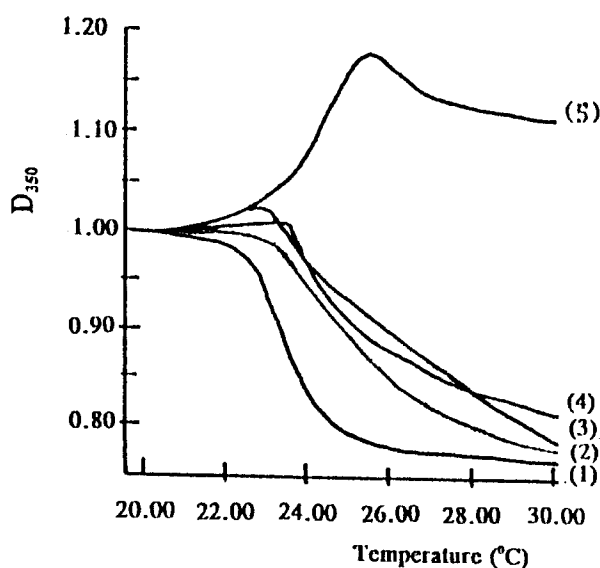


Fig. 1. Influence of GM1 concentration on the interaction of DMPC-liposomes and ricin B-chain (0.03 mg/ml) at the phase transition temperature. (1) DMPC-liposomes without GM1; (2) 2 M% GM1; (3) 4 M% GM1; (4) 8 M% GM1; (5) 10 M% GM1.

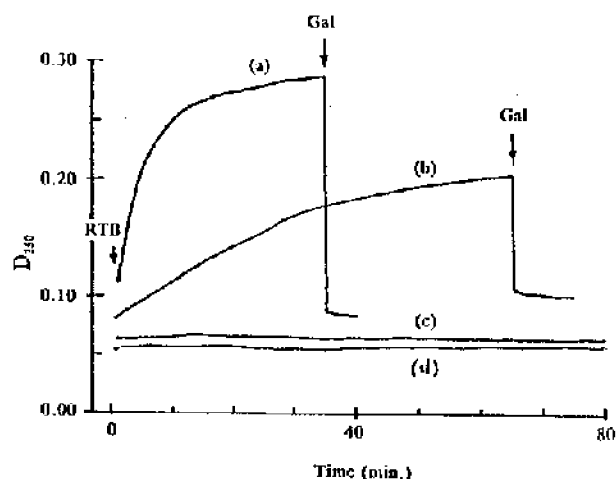


Fig. 2. Influence of ricin and MLI B-chain on DMPC-liposome aggregation: (a) DMPC-liposomes with 10 M% GM1+0.06 mg/ml ricin B-chain; (b) DMPC-liposomes with 10 M% GM1+0.03 mg/ml ricin B-chain; (c) DMPC-liposomes without GM1+0.03 mg/ml ricin B-chain; (d) DMPC-liposomes with 10 M% GM1+0.06 mg/ml MLI B-chain. The arrow indicates addition of ricin B-chain; 'Gal' indicates addition of galactose (50 mM).

native MLI. The amount of binding sites and association constant with Jurkat cells was the same as previously shown [14].

The fluorescence method has been used previously to investigate the interaction of ricin and its isolated subunits with GM1-containing model membranes [7,15]. This indicated that protein-liposome interaction evokes changes in the parameters of both intrinsic protein fluorescence and fluorescence of the covalently bound dansyl. The association constants were different for intact ricin and its subunits and also depended on the liposome composition and pH. It was demonstrated that the interaction of ricin subunits with liposome is accounted for not only by receptor centers but also by other hydrophobic regions of ricin that are inaccessible in the native toxin and

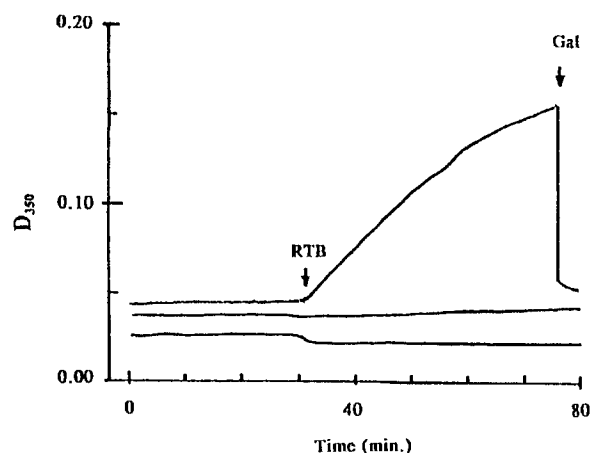


Fig. 3. Influence of ricin A-chain on liposome aggregation: (a) liposomes+0.03 mg/ml ricin B-chain; (b) liposomes+0.03 mg/ml ricin A-chain+0.03 mg/ml ricin B-chain, pH 7.5; (c) liposomes+0.2 mg ricin A-chain+0.03 mg/ml ricin B-chain, pH 5.5. The arrow indicates addition of ricin B-chain; 'Gal' indicates addition of galactose (50 mM).

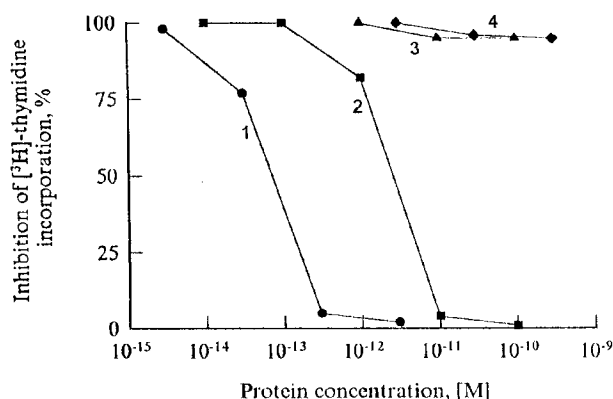


Fig. 4. Cytotoxic activity of ricin (1, 3) and MLI (2, 4) on Jurkat cells in the presence of 50 mM galactose (3, 4) or without (1, 2).

may represent the hydrophobic interface between A- and B-subunits [15].

Our study of the effect of ricin B-chain on the phase transition of DMPC-liposomes at GM1 concentrations of 2, 4 and 8 M% revealed that the increase in the proportion of ganglioside does not give rise to significant changes in absorbance of the liposome suspension. At 10 M% GM1 concentration, a sudden increase in the D350 was observed, corresponding to the onset of aggregate formation. It appears the clustering of the GM1 molecules has taken at the 10 M% concentration of glycolipid [16,17]. In this condition the ricin B-chain can bind to GM1-containing liposomes using both galactose-binding centers. Ricin B-chain would consequently be able to bind to a cell in vivo using both galactose centers [10,18].

The present studies suggest that in the case of ricin B-chain, in addition to the galactose-binding sites, other B-chain domains also interact with liposomes. A possible candidate for this B-chain site is the hydrophobic region participating in the formation of the A-chain–B-chain interface in the native toxin and would therefore not be available for liposome interaction in the case of intact heterodimeric ricin [2]. This idea is supported by our observation that liposome aggregation did not take place when ricin B-chain is present with A-chain in equimolar quantities. Also native ricin did not induce liposome aggregation because the A–B hydrophobic interface was not available for the required interaction with lipids.

In this article model experiments have been carried out for the investigation of two types of RTB interaction with lipid bilayers: (1) carbohydrate binding centers; (2) hydrophobic domain. Such interaction can take place in a cell after reduction of the S–S bond and consequent RTA–RTB dissociation. Ricin A-chain can induce liposome fusion [4]. It was not clear how different cell membrane structures come close in the place of toxin localization. In our paper it has been demonstrated that the main ricin B-chain ‘helper’ function [3,10] is the creation of corresponding conditions for membrane fusion. It is important to point out that it happens after interchain S–S bridge reduction. The improved cytotoxic activity of ricin compared to MLI on Jurkat cells can be explained by the

unique ability of RTB to aggregate model phospholipid vesicles. A-subunits of the toxins have approximately the same enzymatic activity [19] and structural stability [20]. MLI cannot aggregate liposomes because of low hydrophobicity and/or inability to interact with receptors by two carbohydrate-specific centers. Also the cytotoxic action of RIP II proteins is greater than RIP I owing to the existence of a binding subunit that can not only bind to receptor but also obligate A-chain passage to cytosol by interaction with intracellular membranes.

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